Lichao Jiao[a], Xiaoli Liu[a], Xiaomei Jiang and Yafang Yin*

Extraction and amplification of DNA from aged and archaeological *Populus euphratica* wood for species identification

Abstract: The wood samples of *Populus euphratica* Oliv. (Salicaceae) are common archaeological plant remains in the hot and arid regions of western China. However, it is difficult to identify *P. euphratica* wood based on traditional wood anatomical methods alone. DNA barcoding might provide a higher security for species identification. In this study, aged wood specimens stored for approximately 30, 60, and 80 years and archaeological wood up to 3600 years old were in focus to explore the potential of DNA extraction and PCR amplification for different-sized fragments, ranging between 100 and 800 bp, taken from wood stored for different periods. The results indicated that DNA fragments of more than 100 bp could be successfully retrieved from a wood specimen stored for about 80 years based on a modified Qiagen kit protocol. However, it was impossible to obtain DNA segments from the 3600-year-old wood according to the current extraction protocol. Moreover, it was deduced that two-stage PCR amplification could play a significant role in the analysis of DNA retrieved from aged wood materials. With the aid of phylogenetic analysis, based on the short DNA barcode *rbcL*-2 of 202 bp in length, it was possible to differentiate *P. euphratica* from the other species of the *Populus* genus.

Keywords: archaeological wood, chloroplast DNA, DNA barcoding, nuclear DNA, phylogenetic analyses, *Populus euphratica*

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[a]These authors contributed equally to this work.
***Corresponding author: Yafang Yin,** State Key Laboratory of Tree Genetics and Breeding, Research Institute of Wood Industry, Chinese Academy of Forestry, Beijing 100091, P.R. China, e-mail: yafang@caf.ac.cn
Lichao Jiao and Xiaomei Jiang: Wood Anatomy and Utilization Department, Research Institute of Wood Industry, Chinese Academy of Forestry, Beijing 100091, P.R. China
Xiaoli Liu: Beijing Museum of Natural History, Beijing 100050, P.R. China

Introduction

Populus euphratica Oliv. (Salicaceae) is the dominant tree species in the desert regions of western China, Central Asia, and Mediterranean coastal countries, and it is one of the most salt- and drought-tolerant tree species. In the region of western China, the fragments of *P. euphratica* are frequently found as archaeological plant remains; the doubtless identification of which is important in archaeological research. However, it is often impossible to distinguish *P. euphratica* wood from other species of the genus *Populus* based on anatomical features alone. The DNA barcoding technique might overcome these limitations and might provide effective information with a high resolution. DNA barcoding is a genetic approach aimed at achieving accurate species identification based on a standard part of the DNA genome (Degen and Fladung 2007). Currently, the chloroplast genome regions, such as *rbcL*, *matK*, *psbA-trnH*, and the nuclear ribosomal DNA internal transcribed spacer (ITS), have emerged as good candidates for plant DNA barcoding (Kress et al. 2005; Gonzalez et al. 2009; Jiao et al. 2014). In recent years, DNA barcoding has become a reliable tool in the fields of herbal medicinal materials, quality control, forensic science, and the conservation of biodiversity (Li et al. 2011).

DNA extraction from fresh leaves or buds is a matter of routine in molecular biology. However, DNA extraction from dried wood stored for a long-term is more problematic (Finkeldey et al. 2010; Abe et al. 2011; Höltken et al. 2011; Lowe and Cross 2011; Jiao et al. 2012, 2014). After the death of an organism, hydrolytic and oxidative processes cause fragmentation and modification to its DNA (Pääbo et al. 2004; Schlumbaum et al. 2008). DNA increasingly degrades with age, resulting in the splitting of intact DNA into small fragments, ranging from 50 to 500 bp in length (Dumolin-Lapègue et al. 1999; Deguilloux et al. 2002; Speirs et al. 2009; Jiao et al. 2014). Despite all these events, short DNA fragments still contain information that may assist in confirming taxonomic identification and understanding the history of a material.

Because only limited amounts of endogenous DNA survive postmortem degradation in old specimens, the efficiency of DNA isolation and PCR amplification become critical in further DNA analysis. The studies involving the isolation and amplification of DNA in long-term stored wood or ancient wood are still rare. Speirs et al. (2009) reported the amplification of chloroplast DNA regions, smaller than 100 bp, from the oak wood samples taken from a flagship that had been preserved in a marine environment for more than 400 years. Tani et al. (2003) amplified up to 600 bp of nuclear DNA fragments from *Cryptomeria japonica* wood that had been buried in a dam for 3600 years, whereas Deguilloux et al. (2002) demonstrated that it was impossible to retrieve DNA segments of more than 300 bp from the sapwood of oak logs approximately 20 years after felling. Hence, there is a need to explore this field further.

The aims of the present study were (1) to evaluate the feasibility of DNA extraction from aged and archaeological *P. euphratica* wood based on a modified Qiagen kit protocol (Hilden, Germany), (2) to assess DNA preservation by means of amplicon length recovery, and (3) to explore the possibility of *P. euphratica* wood identification based on retrieved DNA barcodes.

Materials and methods

Three aged wood xylarium specimens of *P. euphratica*, viz., w19451, w9998, and w5314, were chosen from the Wood Xylarium Collection at the Chinese Research Institute of Wood Industry in the Chinese Academy of Forestry (Table 1; Figure 1), as well as an archaeological wood specimen of *P. euphratica*, M70, which was taken from a coffin excavated at Xiaohe Cemetery in Lop Nor of Xinjiang (Table 1; Figure 1).

Wood specimen w19451 was excised into small blocks [10 mm (L)×10 mm (R)×10 mm (T)] with a razor blade and then softened in water at 80°C for 5 h. Thereafter, transverse, radial, and tangential sections were cut into thicknesses of 15 μm on a sliding microtome and then observed under a light microscope (Olympus BX61, Japan) after being stained with 1% aqueous safranin.

Table 1 Details of the aged and archaeological wood specimens of *P. euphratica*.

Specimen number	Record date of collection in xylarium	Storage time (years)	Location
w19451	1984	30	Xinjiang, China
w9998	1957	57	Xinjiang, China
w5314	1930–1934	80	Northeast, China
M70	1750 BC–1600 BC[a]	>3600	Xinjiang, China

[a]Xia (2007).

Figure 1 Aged and archaeological wood specimens of *Populus euphratica*. (a) w19451; (b) w9998; (c) w5314; (d) M70.

The exposed surfaces of the wood samples were removed with a sterile scalpel to avoid external contamination. The wood samples weighing 100–200 mg were ground into fine powders in a 6770 Freezer/Mill (Spex SamplePrep, Metuchen, NJ, USA) cooled by liquid nitrogen. All DNA isolations were carried out under sterile conditions. Wood DNA was extracted following the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) protocol with modifications (Rachmayanti et al. 2006; Jiao et al. 2014). Total DNA was subsequently quantified by 1% agarose gel electrophoresis and NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

One-way analysis of variance (SAS program 9.0) was carried out to evaluate the quantitative DNA differences among the samples.

Testing the level of DNA fragmentation in the DNA template extracted from the wood samples (Table 2) was based on five PCR primers, specific to the chloroplast DNA gene for ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*), and one primer, specific to the nuclear ribosomal DNA ITS, ranging in size between approximately 100 and 800 bp.

For the amplification of DNA from the wood samples, two-stage PCRs were carried out. PCR amplifications were carried out with 50 μl of the PCR mixture consisting of 25 μl TaKaRa Premix Ex Taq (containing 1.25 units of Ex Taq DNA polymerase, 2 mM $MgCl_2$, and 200 μM of each deoxyribonucleotide triphosphate; TaKaRa Biotechnology, Dalian, China), 0.4 μM of each primer, and approximately 20 ng of

Table 2 Primer pairs of DNA regions applied for the PCR amplification tests on *P. euphratica*.

Primer name	Sequence (5′-3′)	Annealing temperature (°C)	Fragment length (bp)
rbcL-1	ATCGTTTCTTATTTTGTG CTTTTGATCATTTCTTCG	43	112
rbcL-2	GTTGCTGGAGAAGAAAAT CTTGGATACCATGAGGTG	45	202
ITS	AACGACTCTCGGCAACGG GAGGGTCTCTCAACCACC	54	296
rbcL-3	CGTTTCTTATTTTGTGCC TTCCCCTTCAAGTTTACC	45	366
rbcL-4	GGATTCACCGCAAACACT ATTACCCTCACGAGCAAG	50	510
rbcL-5	GGGTTATCCGCTAAGAAT CACGAGCAAGATCACGTC	47	784

the template DNA. PCRs were performed using Veriti PCR (ABI, Foster City, CA, USA) with an initial denaturing step at 95°C for 5 min, 40 cycles at 94°C for 30 s, at an annealing temperature of 43°C, 45°C, 45°C, 50°C, 47°C, and 54°C for 1 min, respectively, and at 72°C for 1 min. This was followed by a final extension step at 72°C for 10 min. Subsequently, 2 µl of the PCR products were applied as the template for the second amplification with the same primers. Additionally, the negative controls without the addition of wood DNA were carried out in the same amplification protocol. The PCR products were detected in 1% agarose gels afterwards.

The PCR products were purified with the aid of the UNIQ-10 Spin Column DNA Gel Extraction Kit (Sangon, Shanghai, China) and sequenced directly in both directions or sequenced after TA cloning with the *pEASY*-T1 Simple Cloning Kit (TransGen Biotech, Beijing, China) on an ABI PRISM 3730xl (Applied Biosystems, Foster City, CA, USA). When the DNA region was too short for sequencing, TA cloning technology was applied. In this work, the *rbcL*-2, *rbcL*-3, *rbcL*-4, *rbcL*-5, and ITS regions were directly sequenced, whereas the *rbcL*-1 region was sequenced after TA cloning. Three repeated reactions of PCR amplification controls and corresponding sequencings were carried out for each sample to prevent experimental errors, such as DNA polymerase errors during amplification or sequencing errors (Dumolin-Lapègue et al. 1999).

The initial automated alignments of the sequences were obtained with Clustal X 1.81 (Thompson et al. 1997) followed by a manual adjustment using the BioEdit software (Hall 1999). The species discrimination was evaluated with tree-based analyses, which provide a convenient way to visualize the results. A neighbor-joining (NJ) tree was constructed under the P-distance model and pairwise deletion with MEGA 4. The bootstrap values were calculated using 1000 replications in MEGA 4 (Tamura et al. 2007). The taxon assignment success for each barcode, separately and combined, was determined by evaluating the proportion of the monophyletic placements of each species in the NJ trees, including the measures of their clade support.

Results and discussion

Wood anatomy

The wood anatomical characteristics of *P. euphratica* according to the International Association of Wood Anatomists (IAWA) list of microscopic features for hardwood identification (IAWA Committee 1989) are distinct growth rings, diffuse-porous to semi-ring-porous cross-section, solitary vessel outline is angular, radial two to six multiple vessels (or clusters) (Figure 2a); simple perforation plates, mean tangential diameter of the vessels (78±13 µm), absence of helical thickenings, and alternating intervessel pits (Figure 2c); similar vessel-ray pits and intervessel pits (Figure 2b); and the scarcity of parenchyma, uniseriate rays (Figure 2c). The uniseriate rays are 45–496 mm in height and consist of 2–27 (mostly 10–18) cells. All ray cells are procumbent (Figure 2b). However, it is impossible to distinguish *P. euphratica* wood from other species of the genus *Populus*, based alone on wood anatomical characteristics, which are very similar for all species (Cheng et al. 1992).

DNA quantities from aged and archaeological wood specimens

Large quantities of DNA were extracted from specimen w19451 (wood branch stored for more than 30 years). The quantity of DNA was significantly greater than the quantities of DNA obtained from specimens w9998 and w5314 (Table 3). However, there was no significant difference between specimens w9998 and w5314 (Table 3) concerning

Table 3 Analysis of variance in the quantity of DNA (ng mg^{-1} wood) extracted from each wood specimen.

Specimen no.	w19451	w9998	w5314	M70	F-probability
	15.09 A (0.95)	3.76 B (0.33)	3.02 B (0.34)	–	<0.0001

Significant differences among the radial positions are denoted by different letters ($P<0.05$).
The mean values have been calculated from three DNA extractions of each wood specimen, respectively. Data in parentheses are SD. "–" indicates failed to be measured.

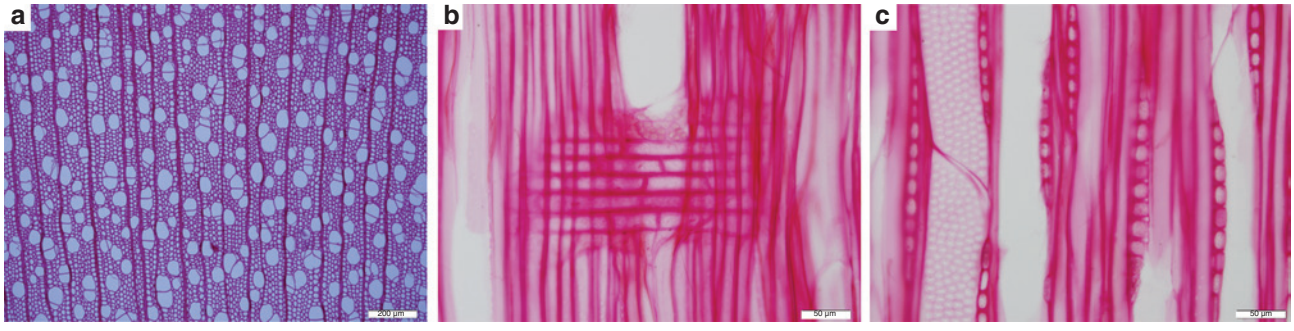

Figure 2 Wood anatomical features of *Populus euphratica* (xylarium w19451 as an example for illustration): (a) transverse section; (b) radial section; (c) tangential section. Scale bars, 200 µm (a) and 50 µm (b and c).

the DNA amounts. Once a tree is cut, the quality and quantity of DNA in the logs will quickly diminish and become degraded into smaller fragments (Bär et al. 1988; Cano 1996; Tnah et al. 2011). The degradation level of DNA becomes relatively stable for several years after felling, although this process does not cease (Speirs et al. 2009).

In the case of specimen M70 that was more than 3600 years old, the quantity of DNA in the extracts was so low that the concentration of the DNA extract could not be measured by UV spectrometry. The wood sample of M70 was soft and its color was brown compared to samples w19451, w9998, and w5314 (Figure 1). A complex aging process involving an oxidative environment and microbial attacks provides an explanation for these observations.

Amplicon length recovery

For the amplification of DNA from wood specimens, two-stage PCRs were carried out successively. After the first reaction, all DNA regions were amplified successfully for DNA extracted from wood specimen w19451. However, no PCR products were obtained from specimens w9998, w5314, and M70. As a result of a second PCR amplification, two short DNA fragments (i.e., *rbcL*-1 and *rbcL*-2) were obtained for DNA extracts from specimen w9998. Furthermore, the shortest DNA fragment (*rbcL*-1), with a length of 112 bp, was successfully amplified for DNA of specimen w5314 (Table 4). However, no PCR products were obtained from DNA extracts of M70 (Table 4). Accordingly, the two-stage PCR amplification was successful for aged woods but not for the 3000-year-old M70. Likewise, Rogers and Kaya (2006) also demonstrated the good performance of a two-stage PCR amplification for DNA retrieved from a specimen of ancient cedar wood.

The DNA solution of M70 was very dark and contained many PCR inhibitors. It is known that purification may enhance the success rate of the PCR. For example, Asif and Cannon (2005) purified DNA samples by means of the High Pure PCR Template Preparation Kit (Roche) and successfully retrieved PCR products. Tani et al. (2003) obtained PCR products from ancient wood DNA by diluting the DNA extracts with water to a ratio of 1:20, which led to inhibitor reduction tolerable to PCR. In the present study, both the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) and a series of dilutions with ratios of 1:5, 1:10, 1:20, 1:30, and 1:40 were used. However, the PCR products could not be amplified successfully. There are two reasons for this observation: (1) the inhibitor levels after purification were still very high and (2) no target DNA fragments were present in the tissues. In the former, the DNA extraction protocol for ancient wood should significantly be improved to minimize the possibility of external contamination (Yang et al. 1997).

Sequence alignment and phylogenetic analyses

The PCR products were sequenced to confirm their identity. When the BLAST search in the National Center for Biotechnology Information nucleotide database was done, the DNA sequences obtained from specimen w19451 mostly matched the sequences of *P. euphratica* deposited in the GenBank, showing a 99% to 100% match with the series of DNA regions. The sequences of *rbcL*-1 and *rbcL*-2 from specimen w9998 displayed a 98% identity with the sequences of *P. euphratica* in the GenBank. There was a 95% match for the sequence of *rbcL*-1 from specimen w5314 (Table 5).

To assess the possibility of barcodes on species discrimination, all single chloroplast DNA barcodes, the above-mentioned barcode combinations, and the ITS barcode were evaluated (Table 6). In the NJ tree based on these barcodes, apart from *rbcL*-1, *rbcL*-3, and the combination of *rbcL*-1 and *rbcL*-3, wood specimen w19451 could be clustered together to form a monophyletic clade with the sequences of the corresponding region of *P. euphratica* from the GenBank (AB012778). Furthermore, it could be separated from the other wood species (Table 6). It should be mentioned that barcodes *rbcL*-1 and *rbcL*-3 did not include enough genetic information for species identification. Moreover, in the NJ tree based on the nuclear DNA region (ITS), specimen w19451 also clustered together

Table 4 Results of the two-stage PCR amplification of DNA barcodes from wood specimens.

	rbcL-1 (112 bp)	*rbcL*-2 (202 bp)	ITS (296 bp)	*rbcL*-3 (366 bp)	*rbcL*-4 (510 bp)	*rbcL*-5 (784 bp)
w19451	√	√	√	√	√	√
w9998	√	√				
w5314	√					
M70						

"√" and empty cell indicate success or failure of PCR amplification, respectively.

Table 5 Maximum match among sequences obtained from wood specimens of *P. euphratica* and sequences of *P. euphratica* deposited in the GenBank.

Specimen no.	DNA region	Maximum match	Accession number
w19451	*rbcL*-5 (756 bp)	99%	AB012778
	rbcL-4 (485 bp)	99%	AB012778
	rbcL-3 (344 bp)	100%	AB012778; KC485209
	ITS (296 bp)	100%	KC485090
	rbcL-2 (202 bp)	100%	AB012778; KC485209; JX571881
	rbcL-1 (112 bp)	100%	AB012778; KC485209
w9998	*rbcL*-2 (202 bp)	98%	AB012778; KC485209; JX571881
	rbcL-1 (112 bp)	98%	AB012778; KC485209
w5314	*rbcL*-1 (112 bp)	95%	AB012778; KC485209

with the sequence of the ITS region of *P. euphratica* deposited in the GenBank (KC485090), which was supported by a bootstrap value of 79% (Table 6). It was reported that the nuclear DNA region (ITS) carried most of the important genetic information and that it had greater mutation rates, compared to chloroplast markers, which was much more preferable for classifying a plant species (Tsumura et al. 2011; Jiao et al. 2014). Similarly, the China Plant BOL Group recommended that the ITS region should be incorporated into the core plant barcode for seed plants, because it shows the highest discriminatory power among the four candidate markers, viz., *rbcL*, *matK*, *psbA-trnH*, and ITS (China Plant BOL Group 2011).

Specimen w9998 could not be discriminated successfully, although specimen w19451 could be grouped together with the sequence of *P. euphratica* from the GenBank (AB012778) in the NJ tree based on the short DNA barcode *rbcL*-2 of 202-bp length or the combination of *rbcL*-1 and *rbcL*-2 (Table 6; Figure 3). More variable sites could be detected in the sequence of *rbcL*-2 from specimen w9998 compared with the *rbcL*-2 sequence of *P. euphratica* from the GenBank (AB012778) and wood specimen w19451 (Table 7). Meanwhile, the most common forms of miscoding lesions for specimen w9998 are the C→T and G→A changes in the amplification products (Table 7), which could be ascribed to the hydrolytic deamination

Table 6 Bootstrap values of monophyletic clades of *P. euphratica* species based on single and combinations of DNA barcodes.

DNA region/specimen	w19451	w9998	w5314
rbcL-1 (112 bp)	n.d.	n.d.	n.d.
rbcL-2 (202 bp)	64	n.d.	–
ITS (296 bp)	79	–	–
rbcL-1+*rbcL*-2 (314 bp)	72	n.d.	–
rbcL-3 (344 bp)	n.d.	–	–
rbcL-1+*rbcL*-3 (358 bp)	n.d.	–	–
rbcL-4 (485 bp)	78	–	–
rbcL-2+*rbcL*-3 (546 bp)	72	–	–
rbcL-1+*rbcL*-2+*rbcL*-3 (560 bp)	74	–	–
rbcL-1+*rbcL*-4 (597 bp)	85	–	–
rbcL-3+*rbcL*-4 (653 bp)	85	–	–
rbcL-1+*rbcL*-3+*rbcL*-4 (667 bp)	86	–	–
rbcL-2+*rbcL*-4 (687 bp)	98	–	–
rbcL-5 (756 bp)	89	–	–
rbcL-4+*rbcL*-5 (767 bp)	87	–	–
rbcL-1+*rbcL*-2+*rbcL*-4 (799 bp)	99	–	–
rbcL-2+*rbcL*-3+*rbcL*-4 (855 bp)	98	–	–
rbcL-2+*rbcL*-5 (958 bp)	99	–	–
rbcL-2+*rbcL*-4+*rbcL*-5 (967 bp)	99	–	–

"n.d." indicates not distinguished and "–" indicates failure of PCR amplification.

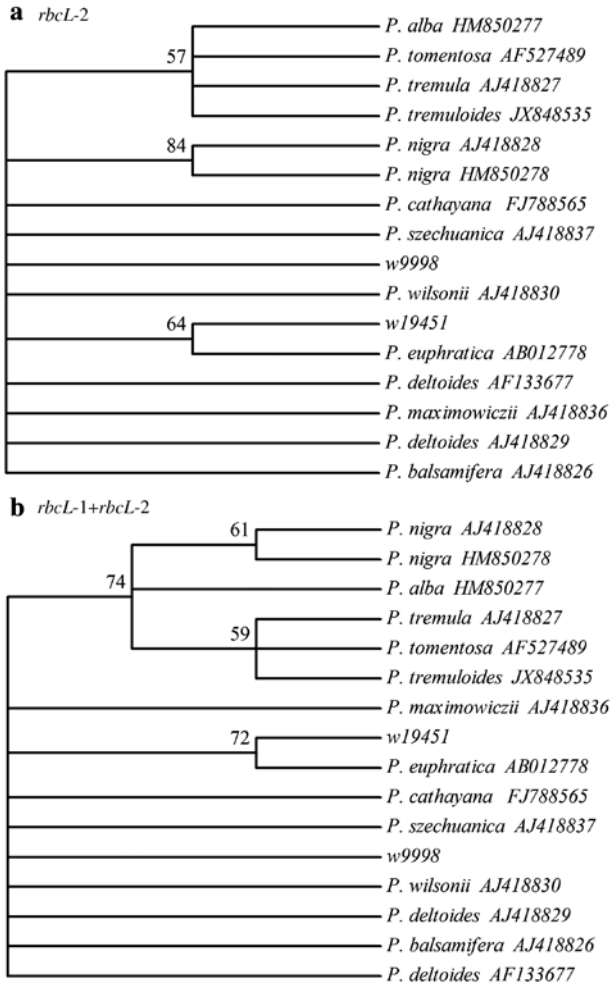

Figure 3 Neighbour-joining (NJ) tree, based on the P-distance of the DNA barcode *rbcL*-2 (a) and *rbcL*-1+*rbcL*-2 (b). Numbers above the branches indicate the percentage of bootstrap values estimated from 1000 bootstrap replicates. Bootstrap values >50% are indicated at the nodes. *P.* = *Populus*.

Table 7 Nucleotide mutations of the *rbcL* region among the GenBank sequences and the wood herbarium specimen of *P. euphratica* that were analyzed.

Taxon	Region	Substitution position													Total sites	Substitution sites
		348	375	403	404	636	643	658	666	687	690	693	702	1017		
AB012778	1–1398	G	G	T	C	C	C	G	C	T	A	C	A	A		
w19451	238–439, 518–1283	–	–	–	–	–	–	–	–	–	–	–	–	**G**	967	1
w9998	238–439, 617–728	**A**	**A**	**G**	**T**	**T**	–	–	–	–	–	–	–	×	314	6
w5314	617–728	×	×	×	×	–	**A**	–	**A**	**C**	**G**	**T**	**G**	×	112	6

Substitutions are indicated in bold, the identical bases are indicated by "–," and "×" indicates no sequencing.

of cytosine to uracil, resulting in a modification of the original sequence information. Staats et al. (2011) also obtained similar results; that is, increased levels of C→T/G→A transitions were observed in the plastid DNA of old herbarium specimens, compared to fresh tissues, representing 21.8% of the miscoding lesions observed. Furthermore, the sequence variations might be caused by the individual variations in the same *P. euphratica* species from different geographic regions.

For specimen w5314, only the short DNA fragment *rbcL*-1 could be amplified successfully. However, specimen w5314 could not be identified, because the DNA region *rbcL*-1 did not include enough genetic information for species identification (Table 6).

A short length of target DNA may lead to a successful PCR but, in general, it does not include enough genetic information for species identification (Yoshida et al. 2006; Abe et al. 2011; Tsumura et al. 2011). Thus, for significantly degraded DNA materials, the barcode regions with short lengths should be selected; they should have high recovery rates (success rates for amplifying and sequencing) and have a lot of potential for species discrimination.

Additionally, the branch support in the NJ tree generally increased when more DNA regions were combined. The combination of some DNA regions received the highest branch support (99%) in the present study (Table 6). In agreement with Liu et al. (2011), it can be stated that a combination of several short DNA barcodes might improve the power of wood species identification.

Conclusion

DNA can be obtained from wood specimens stored for approximately 80 years by using a modified Qiagen kit protocol, whereas DNA fragments of more than 100 bp can be successfully retrieved. DNA information from a specimen that is approximately 3600 years old could not be obtained using this protocol. Dilution and further purification were not helpful, either.

DNA degrades significantly in relation to the length of the function storage time. The size of the amplicon similarly shows a strong correlation with the PCR success, whereas the shortest fragments have the highest success rate. For specimens stored for decades, it might be preferable to focus on short target regions (<300 bp) for amplification. The two-stage PCR amplification technique improves PCR sensitivity and was successful in the present study.

With the aid of phylogenetic analysis, based on barcode *rbcL*-2 (202 bp in length), it was possible to differentiate *P. euphratica* from other species of the *Populus* genus. The nuclear DNA region (ITS) seems to be also suitable for species identification. Furthermore, combining several short DNA regions generally increased the robustness (measured by clade support) of the barcoding discrimination. The feasibility of wood identification based on DNA barcoding method will open new scenarios for the study of wood forensics, certification, archaeology, and paleobotany.

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